In Vivo Studies of Mutant Keratin 1 in Ichthyosis Hystrix Curth–Macklin

To the Editor:

Keratin 1 (K1) is one of the major keratins expressed in differentiating epidermal keratinocytes. Pathogenic mutations in the K1 gene, *KRT1*, have been demonstrated to underlie several distinct disorders of keratinization, such as epidermolytic hyperkeratosis (bullous congenital ichthyosiform erythroderma) and nonepidermolytic, diffuse palmoplantar keratoderma (PPK) (for review see Ishida-Yamamoto *et al*, 2002). Recently, we have identified a heterozygous frameshift mutation in *KRT1* (1609–1610delGGinsA) in a multigenerational family with ichthyosis hystrix Curth–Macklin (IHCM, OMIM 146590) (Sprecher *et al*, 2001). IHCM is a rare autosomal dominant skin disorder characterized by extensive (nonepidermolytic) hyperkeratosis that involves in some families (Ollendorff Curth and Macklin, 1954; Sprecher *et al*, 2001) predominantly palms and soles, although skin over large joints and the trunk may also be affected. The hyperkeratosis may initially show a striate pattern but often is diffuse, leading to severe flexural contractures, fissures, and sometimes loss of digits. IHCM is characterized by specific ultrastructural abnormalities of the cytoskeleton of suprabasal keratinocytes, including an entangled meshwork of individual keratin intermediate filaments (KIF), which aggregate in cytoplasmic shells instead of forming KIF bundles, perinuclear vacuolization, and the presence of binucleated cells (Anton-Lamprecht *et al*, 1973; Niemi *et al*, 1990; Anton-Lamprecht, 1994; Sprecher *et al*, 2001). The mutation 1609–1610delGGinsA, which occurred in a region encoding the variable tail domain (V2) of K1, was shown to replace most of the wild-type carboxy-terminal sequence, including the structurally and functionally important glycine loop motifs of K1, with an aberrant and truncated peptide of 76 residues.

Interestingly, another heterozygous frameshift mutation in *KRT1* (1628delG) at almost the same location was detected in a family with autosomal dominant striate palmoplantar keratoderma (SPPK, OMIM 148700) (Whittock *et al*, 2002). The predicted molecular consequences of this single nucleotide deletion were comparable with those observed in IHCM, including replacement of the carboxy-terminal glycine-rich V2 tail with a novel, alanine-rich and shorter peptide. Nonetheless, clinical features and underlying ultrastructural abnormalities differed. The hyperkeratosis in SPPK was distributed in a linear or plaque-like pattern, was limited to palms and soles, and much less severe than in IHCM. Electron microscopy revealed only slightly decreased electron density of KIF but attenuation of desmosomal midline structures as well as the outer desmosomal plaques where KIF are inserted (Whittock *et al*, 2002). These features broadly overlap with those observed in SPPK due to mutations in genes encoding the major desmosomal proteins, desmoplakin and desmoglein 1, which presumably led to haploinsufficiency of the faulty proteins (Armstrong *et al*, 1999; Rickman *et al*, 1999; Whittock *et al*, 1999; Hunt *et al*, 2001).

To investigate possible pathomechanisms for either KRT1 mutation, Whittock *et al* (2002) performed *in vitro* transfections with four mutant K1(mK1)-GFP constructs, including the mutants causing IHCM and SPPK. In contrast to wild-type K1 (wtK1) with typical filamentous, cytoplasmic distribution, all tested mutants showed partial nuclear localization, although their newly gained V2 sequences did not contain potential nuclear localization signals. As a similar faulty K1 distribution was also observed for K1 variants truncated before or within the V2 domain, it was proposed that nuclear accumulation of mK1-GFP is attributable to the loss of the wild-type V2 tail domain.

To evaluate the biologic relevance of these observations *in vivo*, we assessed in this study the distribution pattern of mK1 as well as the desmosomal ultrastructure in skin samples of a patient with IHCM by immunohistochemistry and immunoelectron microscopy. We raised a polyclonal rabbit antibody (mK1) against a peptide of 14 amino acids corresponding to the carboxy-terminal end of mK1 anchored by a cysteine (C/LAAGALAAGALEAP) (Ishida-Yamamoto *et al*, 2000). Cornified cells obtained from skin scrapings of an affected individual with IHCM carrying mutation 1609–1610delGGinsA (Sprecher *et al*, 2001) and an unaffected control individual were processed for immunoblotting (Ishida-Yamamoto *et al*, 2000). A specific band of 60 kDa corresponding to the mK1 polypeptide, which is 32 amino acids shorter than wtK1 (molecular weight 65494), was detected in the patient sample but not in the control (**Fig 1*a***). Immunohistochemistry was performed on a skin biopsy sample of hyperkeratotic sole skin (**Fig 1*b–e***). In contrast to control skin, mK1 staining was observed from just above the basal cells throughout all layers of the epidermis, including the stratum corneum. Immunofluorescence analysis revealed extensive colocalization of wtK1 and mK1 in the cytoplasm, but neither protein was detected within the nuclei (**Fig 1*e***). Using antibodies against mK1, wtK1, and desmoglein 1 for immunoelectron microscopy, we confirmed the coexpression of mK1 and wtK1 polypeptides in cytoplasmic KIF (**Fig 1*f***) and association of mK1-positive KIF with the desmosomal plaques (**Fig 1*h***), whereas again no nuclear accumulation of mK1 was observed (**Fig 1*i***). The mK1 and wtK1 positive KIF network appeared abnormal and not bundled (**Fig 1*f,h,i***), differing from thick bundles (tonofibrils) labeled only with wtK1 in the normal control skin (**Fig 1*g***). KIF in the basal cells of the patient were normal. In contrast to SPPK, the ultrastructure of desmosomal plaques as well as the expression of desmoglein 1 and desmoplakin did not reveal any abnormalities in IHCM (**Fig 2**). Specifically, inner plaques and midlines of desmosomes in keratinocytes expressing mK1 were well developed and indistinguishable from a normal control (**Fig 2*i,j***).

This study, together with the detection of mK1 by MALDI-TOF mass spectroscopy (Sprecher *et al*, 2001), unequivocally demonstrate that the predicted mK1 allele leading to IHCM is indeed expressed *in vivo* and not subjected to non-sense-mediated mRNA decay (Frischmeyer and Dietz, 1999). The broad colocalization of mK1 and wtK1 also indicates a normal site- and differentiation-specific expression profile of mK1. Considering the

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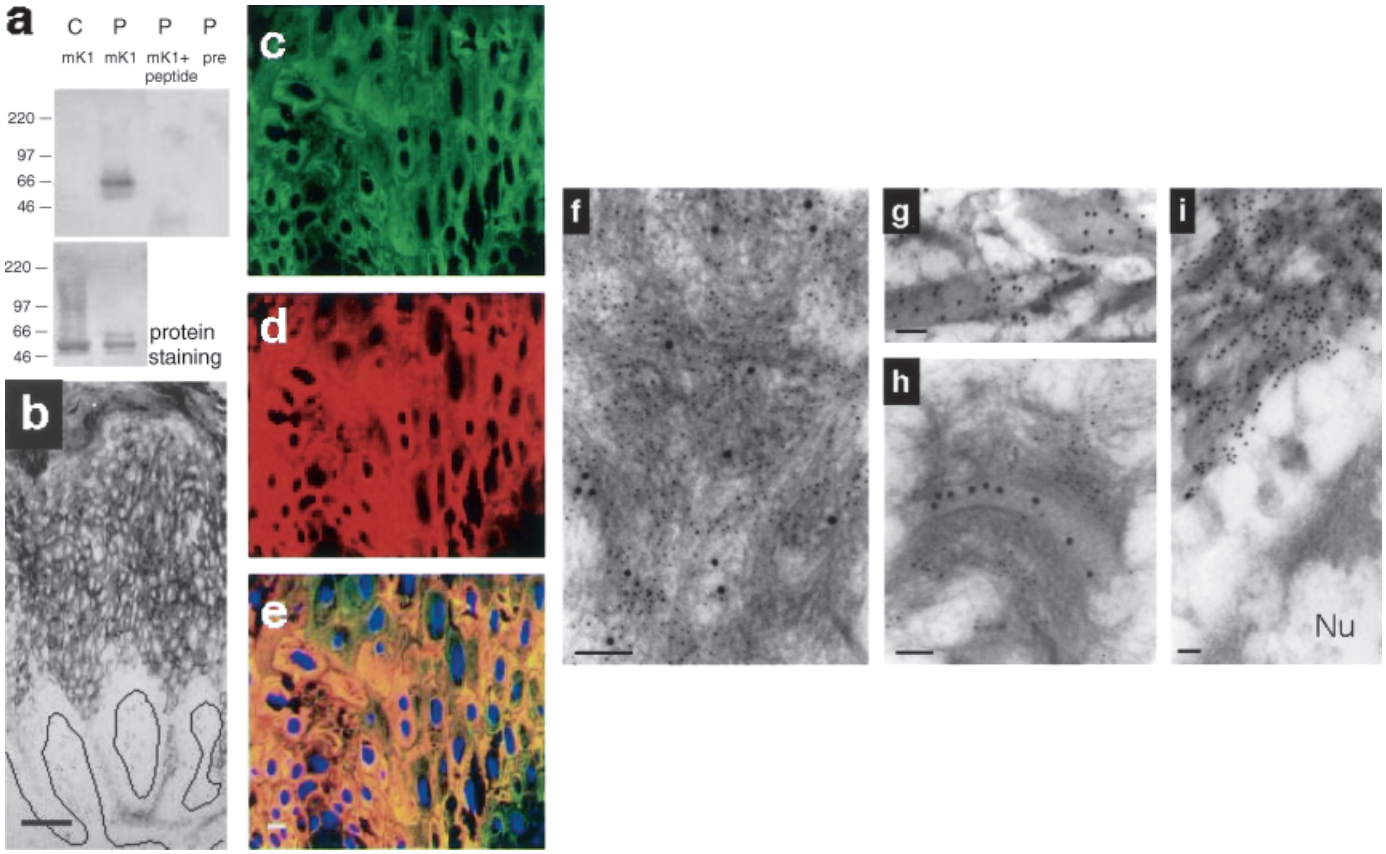

Figure 1. Detection of mK1 in IHCM. (*a*) Immunoblot analysis (*upper panel*). Stratum corneum lysates of the patient (P), and a normal control (C) were loaded, fractionated on 8% sodium dodecyl sulfate–polyacrylamide gels and immunoblotted with mK1, mK1 preincubated with 1 mM mK1 peptide (+peptide) or preimmune serum (pre) (Ishida-Yamamoto *et al*, 2000). The sizes of molecular weight standards are shown on the left. The *arrow* denotes the approximately 60 kDa mK1 protein. Protein staining with Ponceau S (Nacalai Tesque, Kyoto, Japan) of the same membrane is shown in the *lower panel*. (*b*) mK1 immunostaining in lesional plantar skin. Immunogold silver enhancement technique using a section of a Lowicryl K11M resin embedded sample (Ishida-Yamamoto *et al*, 1996). Lines mark basement membranes. (*c–e*) Cryo-ultramicrotome sections of lesional skin (Masunaga et al, 1996) were stained with mK1 visualized with a secondary antibody coupled to Alexa Fluor 488 (Molecular Probes, Eugene, OR) (green), and a wtK1 antibody (34betaB4, Enzo Diagnostics, New York, NY), visualized with a secondary antibody coupled to Cy3 (Amersham Biosciences, Piscataway, NJ) (*red*). (*c,d*) Overlaid on a figure of DNA staining with DAPI (Nacalai Tesque, Kyoto, Japan) (*blue*) shown in (*e*). Digital images were captured using a cooled CCD camera. (*f–i*) Postembedding immunoelectron microscopy (Ishida-Yamamoto *et al*, 1996; Ishida-Yamamoto *et al*, 2000; Sprecher *et al*, 2001). (*f,g*) Double staining of mK1 (visualized with secondary antibody conjugated with 5 nm gold particles) and wtK1 (34betaB4, 10 nm gold particles) of the patient skin (*f*) and normal control subject (*g*). (*h*) Double staining of mK1 (5 nm gold particles) and desmoglein 1 (Dsg1-P23, Progen, Heidelberg, Germany, 10 nm gold particles). (*i*) A nucleus (Nu) is negative to mK1, whereas a shell-like structure surrounding it is positive (10 nm gold particles). *Scale bars*: (*b*) 0.1 mm; (*e*) 0.01 mm; (*f–i*) 100 nm.

abnormal organization of KIF that contain mK1 and their accumulation in shell-like cytoplasmic structures, our observations strongly suggest that this K1 mutant interferes with KIF bundling, possibly in a dominant negative fashion, thus perturbing the KIF cytoskeleton. Our results, however, do not confirm the previously reported *in vitro* data showing nuclear localization of mK1-GFP (Whittock *et al*, 2002). The specific mechanisms leading to trapping and accumulation of mK1-GFP in the nuclei remain unclear. There has been no report showing nuclear localization of keratin proteins *in vivo*. Although the wtK1 construct distributed normally in the transfected cells, the coupled GFP may additionally impair the distribution of mK1 and may therefore lead to the abnormal nuclear localization. Alternatively, a disproportional high expression of K1 constructs could cause aberrant keratin localization. In either case, our data suggest that nuclear keratins do not to play an important part for the disease pathology in the affected skin tissue.

Finally, normal desmosome formation in IHCM suggests that the cytopathologic effects leading to hyperkeratosis and PPK in this disorder differ from those in SPPK, and may be related to abnormalities in supramolecular KIF organization and cytoplasmic trafficking of insoluble proteins, such as loricrin, as previously suggested (Sprecher *et al*, 2001). It is also possible that mK1 impacts upon shapes of the cells and integrity of other cellular structures, such as organelles and the nucleus, as the cells are often binucleated and the granular cells are not flattened (Anton-Lamprecht *et al*, 1973; Niemi *et al*, 1990; Anton-Lamprecht, 1994; Sprecher *et al*, 2001). This may lead to gross alteration in the overall structures of the epidermis.

The reasons for the apparent clinical and ultrastructural differences between IHCM and SPPK despite the similarities of the mK1 products in both disorders are not clear at present. Possible explanations could be slight differences in the length of the abnormal tail domains, expression of other type II keratins that might compensate for abnormal K1 functions, or local factors, such as traumatization due to body weight or manual labor. Future studies of additional families with different mutations in keratin tail domains or transgenic animals expressing this type

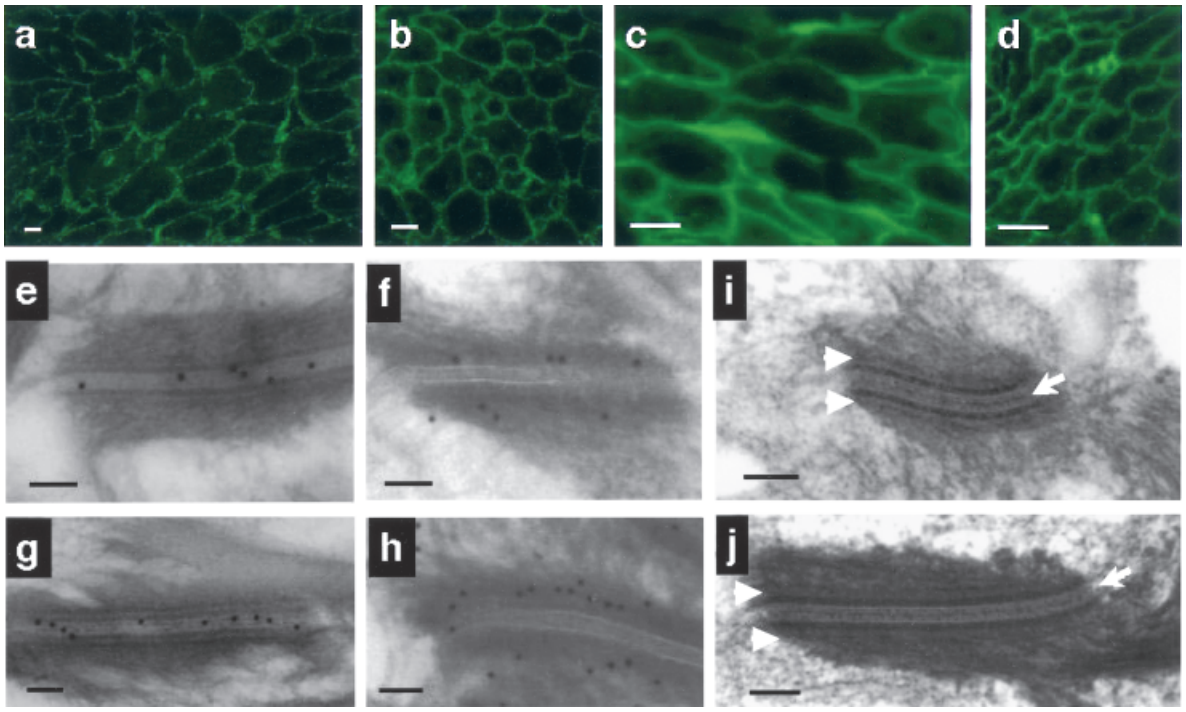

Figure 2. Desmosomes in IHCM. Cryo-ultramicrotome sections were stained with antibodies against desmoglein 1 (*a,b*: Dsg1-P23) and desmoplakin (*c,d*: desmoplakin multiepitope cocktail, American Research Products, Belmont, MA), and detected with secondary antibodies coupled to fluorescein isothiocyanate (DAKO, Glostrup, Denmark). Postembedding immunoelectron microscopy of desmoglein 1 (*e,g*) and cryo-ultramicrotomy staining of desmoplakin. (*f,h*) Ten nanometer gold-conjugated secondary antibodies were used. (*i,j*) Transmission electron microscopy pictures of desmosomes in the superficial epidermis (Sprecher *et al*, 2001). Inner plaques and midlines of desmosomes are marked with *arrowheads* and *arrows*, respectively. Patient skin (*a,c,e,f,i*) and normal sole skin (*b,d,g,h,j*). *Scale bars*: (*a–d*) 0.01 mm; (*e–j*) 100 nm.

of mutations might help to clarify and understand these questions further.

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